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Autoaggregation Response of *Fusobacterium nucleatum*▽†

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Fusobacterium nucleatum **is a gram-negative oral bacterial species associated with periodontal disease progression. This species is perhaps best known for its ability to adhere to a vast array of other bacteria and eukaryotic cells. Numerous studies of *F. nucleatum* have examined various coaggregation partners and inhibitors, but it is largely unknown whether these interactions induce a particular genetic response. We tested coaggregation between *F. nucleatum* ATCC strain 25586 and various species of *Streptococcus* in the presence of a semidefined growth medium containing saliva. We found that this condition could support efficient coaggregation but, surprisingly, also stimulated a similar degree of autoaggregation. We further characterized the autoaggregation response, since few reports have examined this in *F. nucleatum*. After screening several common coaggregation inhibitors, we identified L-lysine as a competitive inhibitor of autoaggregation. We performed a microarray analysis of the planktonic versus autoaggregated cells and found nearly 100 genes that were affected after only about 60 min of aggregation. We tested a subset of these genes via real-time reverse transcription-PCR and confirmed the validity of the microarray results. Some of these genes were also found to be inducible in cell pellets created by centrifugation. Based upon these data, it appears that autoaggregation activates a genetic program that may be utilized for growth in a high cell density environment, such as the oral biofilm.**

Fusobacterium nucleatum is a gram-negative, anaerobic, oral commensal bacterium that is among the most frequently detected species in human subgingival plaque samples of both healthy and diseased sites (29, 32). However, during the progression from oral health to periodontitis, the proportion of *F. nucleatum* increases substantially, which has implicated this species as a contributor to the development of periodontal disease (9, 36, 39). Currently, the mechanisms of *F. nucleatum* pathogenicity remain unclear. Butyrate production has been suggested to be a virulence factor (4), although clinical data suggest that pathogenicity is strain specific and not inherent among the various *F. nucleatum* subspecies (29, 33). Thus, it has been hypothesized that the association of *F. nucleatum* with periodontal disease could be due to its role as a "bridge species" for periodontal pathogens (23).

The term "bridge species" specifically refers to the proposed role of certain bacteria as the interface between the early and late colonizers of the dental plaque (23). Dental plaque is a richly diverse multispecies biofilm composed of both gram-positive and gram-negative bacteria. The specific organization within the oral biofilm community is readily apparent, and plaque development occurs in largely discreet steps. The early colonizers are primarily composed of gram-positive species of *Streptococcus* and *Actinomyces*, which both possess various adhesins that facilitate attachment to the exposed salivary pellicle (21, 34). In contrast, the late colonizers are predominantly gram-negative and are generally not known to adhere to pellicle proteins (34). In addition, a subset of the late colonizers consists of the pathogenic species most often associated with periodontitis, such as *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Porphyromonas gingivalis*, *Treponema denticola*, and others. These and many other late colonizers are thought to rely upon bridge species such as *F. nucleatum* to facilitate their entry into the plaque community (34). This could occur through one of the more peculiar features of *F. nucleatum*: its promiscuous adherence ability. *F. nucleatum* is perhaps best known for its ability to adhere to an extremely diverse array of both prokaryotic and eukaryotic cells (2, 12, 15, 22, 24, 26, 28). When this interaction occurs between planktonic bacterial cells, it is referred to as coaggregation and *F. nucleatum* coaggregates tenaciously with a wide variety of both early and late colonizers (6). Consequently, *F. nucleatum* may serve as a bridge between species that can colonize an exposed tooth surface (early colonizers) and species that require interactions with other species (late colonizers) (23). Since it is the late colonizers that tend to be the species associated with periodontal destruction, bridging from *F. nucleatum* could play a significant role in determining the pathogenicity of the mature oral biofilm community.

Much of what has been published regarding coaggregation has been focused upon identifying coaggregation partners, as well as molecular inhibitors of those interactions. The evidence suggests that strain specificity dictates adhesive properties and thus which coaggregation inhibitors are effective (38, 40). Also, some strains of *F. nucleatum* are sensitive to different inhibitors, which suggests that more than one adhesin may be utilized within a given strain (38, 40). Considerably less has been published regarding the cellular response of *F. nucleatum* to coaggregation, which prompted our interest to determine whether cell-cell adherence impacts its gene regulation. In the

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process of examining coaggregation with species of oral *Streptococcus*, we found that *F. nucleatum* ATCC strain 25586 was similarly proficient at autoaggregation. Therefore, we began by characterizing the autoaggregation ability of 25586 in order to examine whether *F. nucleatum* responds to cell-cell contact with its own species. Our data suggest that autoaggregation, like streptococcal coaggregation, is lysine inhibitable. Furthermore, autoaggregation elicits a surprisingly rapid and robust global transcriptional response, which may be a part of its adaptation mechanism to transition from planktonic growth to persistence in the high cell density environment of the oral biofilm.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *F. nucleatum* subsp. *nucleatum* strain 25586 was purchased from the American Type Culture Collection. Bacteria were grown at 37°C anaerobically (85% N_2, 10% CO_2, and 5% H_2) and maintained in reduced Columbia broth or on Columbia broth agar plates. For experiments measuring aggregation, bacteria were incubated in a semidefined medium (SDM) consisting of: 13.6 mM L-glutamic acid, 1.1 mM L-cysteine, 1.1 mM L-leucine, 1 mM L-lysine, 1 mM arginine, 50 μM L-glycine, 50 μM L-proline, 70 μM adenine, 11.9 mM K_2HPO_4, 21.5 mM KH_2PO_4, 10 mM NH_4Cl, 5 mM urea, 5.5 mM sodium pyruvate, 5.9 mM $MgCl_2$, 0.1 mM $MnCl_2$, 0.1 mM $FeSO_4$, 1 mM $CaCl_2$, 1 mg of riboflavin/ml, 0.5 mg of thiamine/ml, 0.1 mg of D-biotin/ml, 1 mg of nicotinic acid/ml, 0.1 mg of *p*-aminobenzoic acid/ml, 0.5 mg of D-pantothenic acid/ml, 1 mg of pyridoxine/ml, 0.1 mg of folic acid/ml, 0.5% (wt/vol) glucose, and 1% (wt/vol) proteose peptone. In some cases, the SDM also contained 25% (vol/vol) stimulated, cleared saliva. Saliva samples were collected and pooled from five subjects by using a protocol approved by the University of Oklahoma Health Sciences Center Institutional Review Board.

Cell precipitation assay. Stationary-phase cultures in Columbia broth were used as starter cultures. The cultures were diluted 1:100 in SDM and grown overnight for ~16 h to late log phase (optical density at 600 nm [OD_{600}] of 0.7 to 0.9). These cells were then centrifuged and resuspended in fresh SDM. For assays measuring aggregation in the presence of various concentrations of saliva, cells were resuspended in SDM to an OD_{600} of 2.0. Next, 250 μl of this cell suspension was diluted with 750 μl of various combinations of saliva and SDM, which yielded a final OD_{600} of 0.5 to 0.6. For assays measuring the effect of aggregation inhibitors in the presence of saliva, cells were resuspended in SDM to an OD_{600} of ~0.715. Next, 700 μl of this suspension was then diluted with 250 μl of saliva plus 50 μl of the various inhibitors, which yielded a final OD_{600} of 0.5 to 0.6. For all of the cell precipitation assays, the cells were incubated in plastic cuvettes. At the start of the assay, the cells were briefly vortex mixed and measured for their initial ODs. After this initial measurement, the suspensions were incubated at 37°C and measured at regular intervals for a total assay time of 90 min. A decrease in OD over time was indicative of cell aggregates precipitating to the bottom of the cuvette. For all assays, each data point was the average of three independent samples.

RNA extraction. To obtain RNA samples for microarray, cells were prepared as follows. Cells were first grown to stationary phase in Columbia broth, which was then used as a starter culture to inoculate SDM cultures. Stationary-phase cells were diluted 1:100 in 20 ml of SDM and grown 16 h overnight to late log phase (OD_{600} of 0.7 to 0.9). The following day, the cells were centrifuged and resuspended to an OD_{600} of 0.5 to 0.6 in either 25 ml of SDM, SDM plus 25% saliva, or SDM plus 25% saliva and 50 mM L-lysine. Next, the cells were prewarmed to 37°C before incubating anaerobically for 90 min. at 37°C. Every 15 to 20 min., the nonaggregating cultures (SDM and SDM plus saliva/lysine) were gently agitated to maximize separation between the cells and prevent any settling. After the assay period, the cells were immediately centrifuged at 4°C in a prechilled refrigerated centrifuge. Cell pellets were resuspended in 500 μl of chilled Tris-EDTA buffer on ice. Next, the cells were placed in a 70°C water bath, and 900 μl of preheated acidic phenol (pH 4.3) was immediately added to the cultures, followed by sodium dodecyl sulfate to a final concentration of 1% (wt/vol). The mixture was incubated for 5 min before removal from the water bath, addition of 400 μl of chloroform, and centrifugation. The mixture was further phenol-chloroform extracted three times before the RNA was precipitated and resuspended in 90 μl of RNase-free water plus 10 μl of DNase buffer. DNase was added to the samples, followed by incubation at 37°C for 45 min. Finally, the samples were further purified with Qiagen RNeasy MinElute spin columns according to the manufacturer's protocol and eluted in RNase-free water. The resulting RNA integrity was confirmed by the presence of clearly defined rRNA bands in agarose gels and later quantified for concentration by OD_{260} readings.

Microarray. The cDNA used for microarray experiments was prepared similarly, as suggested by the GeneChip expression analysis technical manual (Affymetrix, Santa Clara, CA). The protocol is briefly summarized here. First, 15 μg of RNA was mixed with 1.25 μg of random hexamers and heated at 70°C for 10 min to denature the sample before the addition to the reverse transcription (RT) reaction. RT was performed in a total volume of 60 μl and contained 12 μl of 5× Superscript II buffer (Invitrogen, Carlsbad, CA), 6 μl of 100 mM dithiothreitol, 3 μl of 10 mM deoxynucleoside triphosphate, 1.5 μl of RNaseOUT RNase inhibitor, and 7.5 μl of Superscript II reverse transcriptase. The reaction was incubated at 42°C for 60 min and then terminated by incubation at 70°C for 10 min. RNA in the mixture was then hydrolyzed by adding 15 μl of 1 N NaOH and incubating the reaction at 65°C for 30 min. The solution was neutralized with 1 N HCl, and the cDNA was then purified by using a Qiagen MinElute PCR purification kit. The concentration of cDNA was determined by measuring the absorbance at 260 nM and 3 μg of cDNA was fragmented in a 35-μl reaction containing 3.5 μl of Roche 10× DNase buffer and 7.5 μl of diluted Roche DNase (0.06 U/μl). The fragmentation reactions were prepared on ice and then incubated at 37°C for 10 min before terminating the reaction by incubation at 98°C for 10 min. Next, 32 μl of the fragmented cDNA was finally labeled with a BioArray terminal labeling kit (Enzo, New York, NY) in a total volume of 50 μl containing 10 μl of 5× buffer, 5 μl of 10× $CoCl_2$, and 2 μl of terminal deoxynucleotide transferase. The reaction was incubated at 37°C for 60 min.

Labeled cDNA was used for hybridization to a standard 49 format custom GeneChip microarray (Affymetrix) containing each of the predicted open reading frames of *F. nucleatum* ATCC strain 25586. The probe selection and GeneChip design were performed in conjunction with Affymetrix. In addition, the microarray incorporates all of the control elements recommended by Affymetrix for the determination of background signal intensity and hybridization specificity. The hybridization and washing procedures were performed similarly as recommended in the GeneChip expression analysis technical manual and as previously described (1) with some minor modifications. The hybridization buffer consisted of 1× MES buffer (100 mM morpholineethanesulfonic acid [MES], 1 M NaCl, 20 mM EDTA, 0.01% Tween 20), 50 pM B2 control oligonucleotide, 0.1 mg of herring sperm DNA/ml, 0.5 mg of bovine serum albumin (BSA)/ml, and the fragmented, labeled cDNA in a total volume of 200 μl. The hybridization solution was then loaded into the microarray cartridge and mixed for 16 h at 60 rpm and 47°C in an Affymetrix hybridization oven. After the incubation period, the hybridization solution was removed, and the array was washed and stained in the Affymetrix Fluidics Station 450 using a slightly modified script of the standard wash script file "ProkGE-WS2v3-450," which increased the wash stringency from 45 to 47°C. The staining solution consisted of three different MES buffers each in a 600-μl total volume. The first consisted of 1× MES buffer, 2 mg of BSA/ml, and 10 μg of streptavidin/ml; the second consisted of 1× MES buffer, 2 mg of BSA/ml, 0.1 mg of goat immunoglobulin G/ml, and 5 μg of biotin anti-streptavidin/ml; and the third solution consisted of 1× MES buffer, 2 mg of BSA/ml, and 10 μg of streptavidin-phycoerythrin (Molecular Probes, Eugene, OR)/ml. The GeneChips were scanned at 570 nM by using an Affymetrix 7G laser scanner.

Analysis of signal intensities was performed by using the GeneChip operating system software (GCOS) version 1.4, and gene expression data were compared using the GCOS batch analysis function. Normalization procedures are performed directly by the software using a script designed by Affymetrix and provided with the *F. nucleatum* custom array. This eliminates user bias during the normalization procedure. A total of four separate aggregation microarray experiments using two different assay conditions were performed: two independent sets measuring saliva versus no saliva and two independent sets measuring lysine inhibition versus no inhibition. The data from all four experiments were compared, and shared gene responses between the experiments were considered to be due to the effect of cell aggregation. Gene responses that were unique to either microarray condition were considered to be unrelated to autoaggregation and were removed from the final data set. Gene responses that averaged twofold or higher were considered significant.

RT-PCR. Real-time reverse transcription-PCR (RT-PCR) was performed to validate the results generated from the microarray analysis. From the list of ≥2-fold gene responses, representative genes were chosen for further analysis to sample a variety of gene functions, chromosomal locations, and transcriptional responses. Primers were designed by using Applied Biosystems Primer Express 3.0 software, which scans DNA sequences for primers suitable for $\Delta\Delta C_T$ analysis. The primer sequences are listed in Table 1. Cells were grown, and RNA was extracted as described above. A total of 300 ng of RNA was reverse transcribed

TABLE 1. Primers used for real-time RT-PCR analysis

Primer	Sequence (5′–3′)	Target
Fnuc 16S F	TGCAAGTCTACTTGAATTTGGGTTT	16S rRNA
Fnuc 16S R	CTAGCTGTGAGGCAAGTTCTTTAC	16S rRNA
Fn0078 RT-f	AGCAGGAATGACTCCCTCATCTAT	FN0078
Fn0078 RT-r	GATTTCCTTGCAGTTTCTCCTGTT	FN0078
Fn0085 RT-f	TTTTAACACCATCAGCTAAGCAATTT	FN0085
Fn0085 RT-r	TTTCTTCTGCTTCAACATTATCTTCTTC	FN0085
Fn0271 RT-f	AGAAGTTAATATGGATGGCTTAATTGG	FN0271
Fn0271 RT-r	TGCTGCACCACAAACTGAAGT	FN0271
Fn0650 RT-f	GTGGTGGCTTACAATCAATGATGT	FN0650
Fn0650 RT-r	CACCAGTTTTTTCTAATATTCCTCCAT	FN0650
Fn0796 RT-f	GGACATAGAGGATGTAGACTTGGTGTAA	FN0796
Fn0796 RT-r	TTTGCACATTCATATGCTGCTTCT	FN0796
Fn0941 RT-f	GCAGCTGGTTTTGCTTTAGGA	FN0941
Fn0941 RT-r	GCATAAAACCTCCACCACCTAGA	FN0941
Fn1586 RT-f	GTATGGCTGAAATAGTTGGTGTTGA	FN1586
Fn1586 RT-r	TGCTGCATTTACACTAACTTTTGCT	FN1586
Fn1803 RT-f	CTGATGATATTACTGGACAGACTGATCTC	FN1803
Fn1803 RT-r	GCTTCTTCCTCTAAAATACTTCCTTCTTT	FN1803
Fn1900 RT-f	ACAGGTATGTATGGTGCAGGAAGTAT	FN1900
Fn1900 RT-r	CCATTGGTTCGCCAACTTTT	FN1900
Fn1988 RT-f	AAGAAGAATTCCCAGCTCAAGCT	FN1988
Fn1988 RT-r	TCCATAGTTCTTACACCACATTCAACA	FN1988
Fn1989 RT-f	CAGGTGCCATTGAAGGATATAAATAC	FN1989
Fn1989 RT-r	TGCATTAATCCAAGTTTCCACATT	FN1989
Fn2077 RT-f	AACTGGTGCTTCTTTTGGAGATAAA	FN2077
Fn2077 RT-r	TGTGGCAGCAGCAAGGTTT	FN2077

using the manufacturer's protocol for Affinityscript reverse transcriptase (Stratagene, La Jolla, CA). Real-time PCR was performed using an Applied Biosystems 7300, and the reactions were prepared using Applied Biosystems SYBR Green PCR Master Mix. Changes in gene expression were calculated automatically in the Applied Biosystems 7300 System software using the $\Delta\Delta C_T$ method as follows: $\Delta C_T = C_T$ (target) $- C_T$ (housekeeping gene); $\Delta\Delta C_T = \Delta C_{T1} - \Delta C_{T2}$. Fold changes were calculated as $2^{-\Delta\Delta}C_T$. The 16S rRNA gene was used as the housekeeping gene reference, and all samples included a no-RT control to assess genomic DNA contamination in the reactions.

Cell pelleting assay. Late log-phase cultures of 25586 grown in SDM were measured for OD, and the equivalent of 25 ml of OD_{600} 0.5 cells was then transferred to new tubes. The cells were centrifuged at 3,000 × *g* for 3 min, and 25 ml of fresh medium was added back to the tubes. One set of tubes was resuspended, while the others were allowed to remain as cell pellets. Pelleted samples received either SDM, SDM plus 25% saliva, or SDM plus 25% saliva plus 50 mM L-lysine. All samples were incubated for 1 h at 37°C before proceeding with RNA extraction and RT-PCR as described above.

Microarray data accession number. Microarray data are available in the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE18554.

RESULTS

Saliva-induced aggregation. In an effort to examine the cell-cell adherence response of *F. nucleatum* subsp. *nucleatum* ATCC 25586, we first sought to find an alternative method to stimulate coaggregation, since the typical coaggregation assay utilizes a salt buffer that does not support cell growth. Given that coaggregation of planktonic cells in the mouth is likely to occur in the presence of saliva, we began by testing coaggregation in saliva diluted with an SDM we had previously optimized for the growth of *F. nucleatum*. We found that this worked very efficiently with multiple species of *Streptococcus*, since visible coaggregates and cell precipitation were readily apparent within a short time frame. Microscopic analysis further confirmed the presence of large aggregates of intermixed fusobacteria and streptococci as well (data not shown). Unexpectedly, we had also noticed that cellular aggregation of single species cultures of 25586 occurred with an efficiency similar to that of the coaggregating samples. Thus, it appeared as if saliva stimulated the ability of 25586 to adhere both to itself and to other species. This was quite surprising since *F. nucleatum* is widely studied for its ability to coaggregate; however, few reports mention its autoaggregation ability (20, 35). To further confirm that the intraspecies aggregation response was stimulated by the presence of saliva, we tested several concentrations of saliva diluted in the same SDM and measured aggregation with a simple cell precipitation assay. Similar to assays of coaggregating bacteria, 25586 single-species aggregates precipitate out of suspension and accumulate over time. Thus, we were able to quantify this effect as a drop in the OD during incubation in plastic cuvettes. As shown in Fig. 1, both 75 and 50% saliva solutions quickly aggregated, and a noticeable

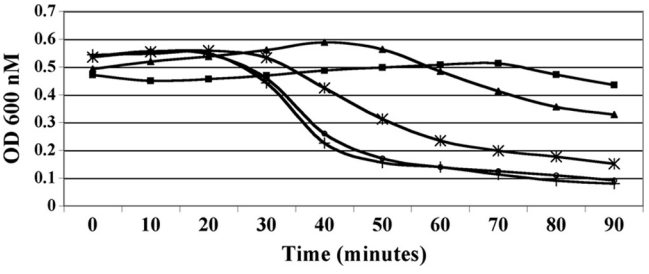

FIG. 1. Effect of saliva concentration on autoaggregation. *F. nucleatum* cells were incubated in SDM containing a range of saliva concentrations and measured for OD every 10 min. The saliva concentrations are as follows: squares, 0%; triangles, 10%; asterisks, 25%; circles, 50%; and crosses, 75%. The results presented here are the average of three independent samples. This experiment was performed three times with similar results.

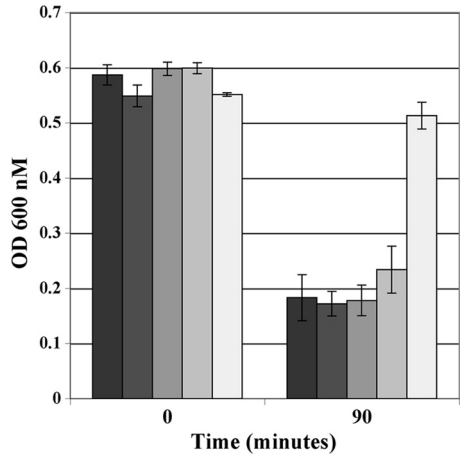

FIG. 2. Effect of 50 mM coaggregation inhibitors on autoaggregation. *F. nucleatum* cells were incubated in SDM containing 25% saliva and several different coaggregation inhibitors. The samples from left to right are as follows: water, lactose, galactose, arginine, and lysine. The results presented here are the averages of three independent samples. This experiment was performed two times, with similar results each time.

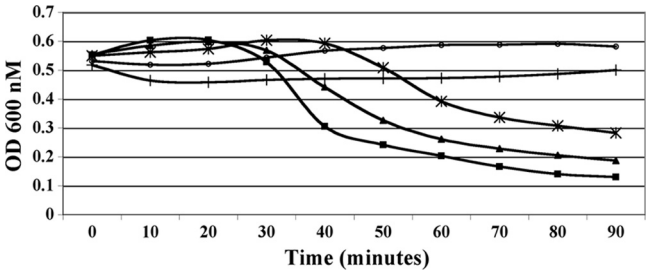

FIG. 3. Effect of lysine concentration on autoaggregation. *F. nucleatum* cells were incubated in SDM containing a range of lysine concentrations and measured for OD every 10 min. The lysine concentrations are as follows: squares, 0 mM; triangles, 6.25 mM; asterisks, 12.5 mM; circles, 25 mM; and crosses, 50 mM. The results presented here are the averages of three independent samples. This experiment was performed three times, with similar results each time.

change in the OD began within 30 min of the start of the assay, although cell aggregation was apparent by visual inspection before the cells started to precipitate. A 25% saliva concentration also induced rapid precipitation, whereas the 10% saliva concentration took noticeably longer and never precipitated as thoroughly as the higher saliva concentrations. The 0% saliva sample remained virtually unchanged throughout the assay, with no evidence of precipitation. However, when the cells were left to incubate for longer periods of time (>2 h), we did observe some aggregation in the absence of saliva (data not shown). A similar phenomenon can also be observed in overnight cultures of 25586, which always exhibit a large degree of precipitation (data not shown). Thus, it appeared that saliva either serves to increase the rate of aggregation, or there are saliva-dependent and -independent mechanisms that can both result in autoaggregation. Furthermore, we repeated the same assay using another strain related at the subspecies level (*F. nucleatum* subsp. *nucleatum* ATCC 23726) and found that this strain lacked the ability to autoaggregate in the presence of saliva (data not shown). Therefore, the autoaggregation stimulatory effect of saliva appeared to be strain specific.

Inhibition of saliva-induced aggregation. Next, we were curious as to whether the aggregation of *F. nucleatum* could be inhibited similarly as has been reported for its numerous coaggregations with other oral bacteria. Generally, coaggregation inhibitors have been identified using various sugars and amino acids (6). Therefore, we tested several commonly used coaggregation inhibitors for *F. nucleatum* and assayed their effect in the presence of SDM supplemented with 25% saliva. As shown in Fig. 2, 50 mM D-lactose and D-galactose had no observable effect upon autoaggregation after 90 min, whereas 50 mM L-arginine tended to slow the aggregation process but could not block precipitation over the course of the assay period. In contrast, the cells incubated with 50 mM L-lysine exhibited no evidence of precipitation, and visual inspection confirmed an evenly turbid culture with no obvious signs of cell aggregation. Hence, it appeared as though lysine was an inhibitor of the autoaggregation ability of 25586. We also found that this inhibitory activity was not absolute, since longer inhibition times (>2 h) still resulted in signs of aggregation (data not shown). Similar to our previous assay of saliva, we decided to determine the concentration dependence of the lysine effect. Since we had already observed that 50 mM lysine was inhibitory in the presence of 25% saliva during our 90 min. assay, we set this as our upper limit and compared the result to 25, 12.5, 6.25, and 0 mM lysine. From the results shown in Fig. 3, there seemed to be the largest difference between the 12.5 and 25 mM lysine samples. At 12.5 mM lysine, the cells precipitate at a rate that is somewhat proportionally slower than with 0 or 6.25 mM. However, this was not the case for 25 mM lysine, since it was largely resistant to aggregation during the assay. Thus, under these conditions, the inhibitory action of lysine may have some critical threshold value between 12.5 and 25 mM. As expected, no evidence of aggregation was visible in the 50 mM lysine samples throughout the course of the assay. We also examined coaggregation between 25586 and several species of oral *Streptococcus* under the same conditions and found that 50 mM lysine was similarly able to inhibit the ability of this strain to bind to other species (data not shown). These results were consistent with a previous study that suggested lysine functions as a streptococcal coaggregation inhibitor for 25586 (38). Therefore, in 25586 it is likely that either the same adhesin is responsible for autoaggregation as well as coaggregation with *Streptococcus* or two separate adhesins are both inhibited by lysine.

Transcriptome analysis of planktonic versus aggregated cells. Given the strong propensity for 25586 to aggregate under the conditions of our assay, our next goal was to determine whether aggregation would elicit any gene responses from the cells. For this purpose, we decided to take a microarray approach to measure the effect of intraspecies cell aggregation upon the transcriptome. Since saliva could induce aggregation and lysine could inhibit it, we could take two potential microarray approaches (Fig. 4). We could either measure the transcriptome of cells incubated in (condition 1) SDM plus saliva (aggregation) versus SDM (planktonic) or (condition 2) SDM plus saliva (aggregation) versus SDM plus saliva plus lysine (planktonic). However, both approaches had the potential

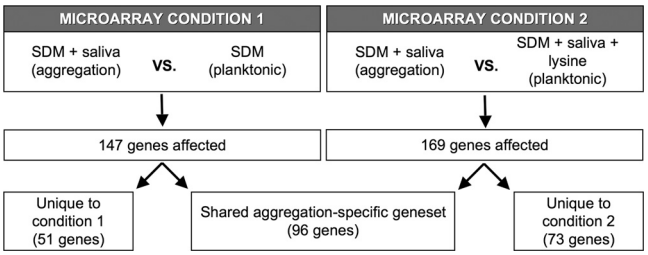

FIG. 4. Subtractive microarray approach to assay autoaggregation. As described in the text, the effect of autoaggregation was determined using two separate conditions: "SDM +/− saliva" and "SDM + saliva +/− lysine." To determine whether a gene is either affected by aggregation or differences in medium composition, the responses from both conditions were compared. Only the gene responses that were similar between the two microarray experiments were considered to be due to autoaggregation, whereas gene responses that were specific to either of the two microarray datasets were attributed to factors unrelated to autoaggregation. Arrows underneath each microarray condition point to the total number of affected genes from each experiment. A comparison of the two datasets identified the shared gene responses, which were attributed to aggregation. The total number of genes within each geneset is listed.

drawback of measuring gene responses to aggregation, as well as differences in medium composition (i.e., saliva for the first approach or lysine in the second approach). In order to circumvent this limitation, we decided to assay both conditions (condition 1 [+/− saliva] and condition 2 [saliva +/− lysine]). We reasoned that if aggregation elicited a specific and reproducible genetic response, then both microarray approaches should yield a shared geneset, since aggregation would occur in both approaches (Fig. 5). In contrast, responsive genes that were uniquely affected in either experiment were most likely attributable to factors other than aggregation, such as differences in medium composition (Fig. 4). Thus, we compared the results from the two microarray experiments using a cutoff value of a ≥2-fold change and were able to identify 96 genes with similar responses in both experiments. In microarray condition 1, this left 51 gene responses that were unlike those of microarray condition 2 and 73 responses in condition 2 that were dissimilar to microarray condition 1 (Fig. 4). From these results, we concluded that the 96 genes similarly affected in both experiments were most likely to be specifically responsive to aggregation, whereas the 51 and 73 unique gene responses in microarray conditions 1 and 2 were probably due to other factors. Consequently, we focused our analysis on the shared geneset of 96 genes. From this group of genes, 54 exhibited changes of ≥3-fold. Interestingly, the vast majority of the 96 aggregation-specific genes were upregulated, while only four genes were downregulated. This result is consistent with the activation of a genetic program. Although we did not detect changes in the transcription of any putative sigma factors, we did identify three putative transcription regulators that were induced, FN0795, FN1439, and FN1803, which may account for some of the observed gene expression changes in the list (Table 2). FN1803 was particularly interesting because its microarray signal intensity in the dispersed samples was quite weak, much lower than FN0795 and approximately half that of FN1439 (see Table S1 in the supplemental material). FN1803

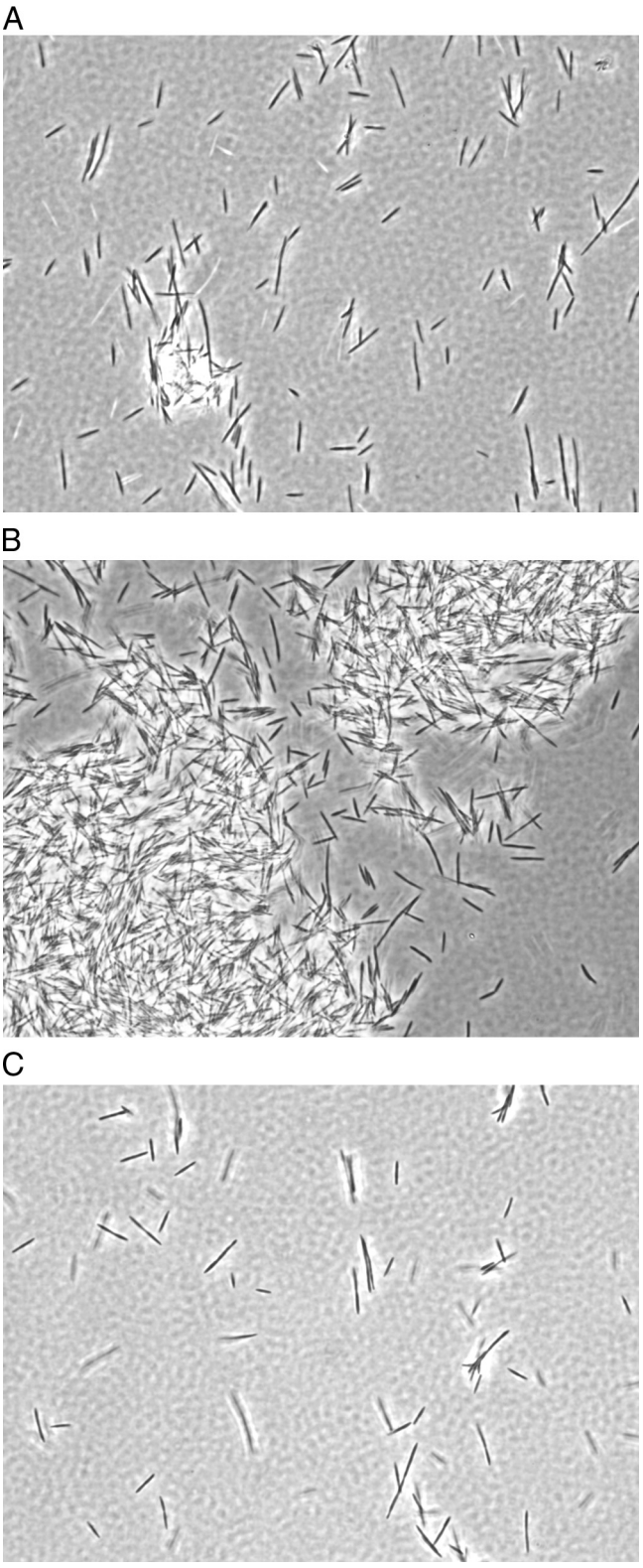

FIG. 5. Phase-contrast images of *F. nucleatum* cells used for microarray. Phase-contrast images of the same cultures used for RNA extraction and microarray were taken just prior to RNA extraction. The total magnification is ×1,000. The medium compositions are as follows: SDM (A), SDM plus 25% saliva (B), and SDM plus 25% saliva plus 50 mM lysine (C).

TABLE 2. Selected genes from the microarray data set[a]

Gene ID	Avg fold change (SD)		Functional class
	Condition 1	Condition 2	
FN0078	8.59 (0.84)	4.57 (1.54)	Ethanolamine utilization
FN0081	7.75 (2.96)	4.9 (2.92)	Ethanolamine utilization
FN0085	4.34 (1.86)	3.0 (1.41)	Ethanolamine utilization
FN0273	7.73 (0.38)	6.73 (0.33)	Butyrate fermentation
FN0516	2.66 (0.52)	1.87 (0.18)	Antimicrobial efflux
FN0553	2.97 (0.72)	3.61 (0.18)	Serine catabolism
FN0591	8.17 (2.37)	5.49 (0.8)	Aminobenzoyl-glutamate transport
FN0650	6.64 (1.92)	8.11 (3.46)	Na^+/H^+ antiporter
FN0796	2.22 (0.11)	2.3 (0.23)	Energy metabolism
FN0941	8.5 (6.36)	5.55 (1.35)	γ-Glutamyltranspeptidase
FN0999	8.96 (5.71)	6.76 (0.99)	Aminopeptidase
FN1186	10.2 (0.5)	3.38 (0.5)	Amidohydrolase
FN1280	2.49 (0.48)	2.49 (0.48)	Peptidase/nuclease
FN1422	4.9 (1.65)	2.89 (0.84)	Na^+/H^+ antiporter
FN1439	3.01 (1.01)	1.41 (0.0)	Transcription regulator
FN1584	6.46 (2.17)	4.33 (0.84)	β-Lactamase
FN1586	4.69 (1.36)	2.97 (0.72)	Aromatic acid catabolism
FN1733	3.98 (2.38)	2.84 (0.24)	V-type sodium ATP synthase
FN1741	2.45 (0.82)	2.07 (0.1)	V-type sodium ATP synthase
FN1803	5.22 (2.46)	3.66 (0.89)	Transcription regulator
FN1900	0.52 (0.07)	0.23 (0.04)	Conserved hypothetical protein
FN1988	22.1 (20.7)	20.2 (13.7)	Tyrosine catabolism
FN1989	24.4 (25.3)	16.1 (11.5)	Tyrosine transporter
FN2077	20.2 (20.0)	16.7 (2.44)	Na^+/H^+ antiporter

[a] Shown here are the average fold changes for the two microarray condition datasets. Only one gene per operon is listed here.

TABLE 3. Summary of affected genes

Function	Total no. of genes
Transport/substrate binding	24
Energy metabolism	20
Hypothetical proteins	18
Central intermediary metabolism	9
Amino acid metabolism	4
Proteolysis	4
Transcription regulation	3
Other	14

also exhibited the greatest increase in expression due to aggregation (three- to fivefold).

The overall response to aggregation is summarized in Table 3. When grouping genes by basic functional categories, the greatest number of genes affected by cell-cell contact involves transport and/or substrate binding. Of these, a large portion comprises putative permeases of a variety of nutrients, as well as putative phosphoenolpyruvate-dependent sugar phosphotransferase system components, which presumably bind certain sugars. Similarly, numerous putative amino acid and peptide transporters were induced as well. In addition to genes involved in nutrient acquisition, three separate putative Na^+/H^+ antiporters were induced (FN0650, FN1422, and FN2077), as well as an operon predicted to encode an efflux pump used to confer resistance to multiple antimicrobials (FN0515 and FN0516). Given the large number of transporters affected, it is not surprising that many genes involved in energy and/or intermediate metabolism were affected as well. From these genes, a large portion (at least 10) can be attributed to the metabolism of ethanolamine. The genes required for this ability are all clustered and arranged into two to three large operons of "*eut*" genes (FN0078 to FN0090).

Real-time RT-PCR confirmation of microarray results. Based upon the microarray data, it appeared that aggregation had a similar effect upon gene expression when measured under both assay conditions (+/− saliva and saliva +/− lysine). To further confirm these data, we analyzed the RNA used for the microarray studies via real-time RT-PCR. We tested a representative subset of the total microarray data set based upon chromosome location, putative gene function, and the magnitude of the gene expression change. As shown in Fig. 6, we found that most of the genes were indeed affected quite similarly between the +/− saliva and saliva +/− lysine experiments. The exception was FN1900, which was one of only four genes found by microarray to have lower expression upon aggregation. From the real-time RT-PCR analysis, it appeared that this gene is lower expressed only in the saliva +/− lysine condition, which suggests that this effect is probably unrelated to aggregation. However, it should be noted that in the microarray data set, the decreased expression of FN1900 was much more apparent in the saliva +/− lysine samples (>4-fold) compared to the +/− saliva samples (<2-fold) (Table 2). Therefore, the trend in FN1900 gene expression was still consistent between the microarray and real-time PCR analyses. Overall, we found that the degree of differential gene expression in the real-time PCR analysis corresponded reasonably closely to the microarray values with the main exceptions being FN1988 and FN1989, which seemed to have been underestimated by microarray analysis. However, the trends of expression were again consistent, since these genes had the greatest increases in both the microarray data set and the real-time PCR experiments. In addition, there seemed to be little evidence that the microarray had overestimated gene expression changes. Thus, it appears that the microarray data are likely to

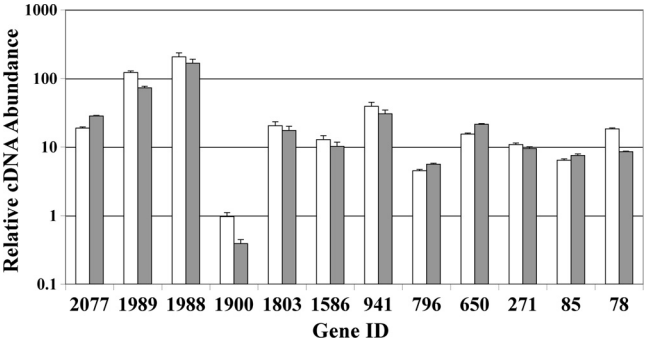

FIG. 6. Real-time RT-PCR of selected genes from the microarray. Twelve genes identified from the microarray data were assayed by real-time RT-PCR. cDNAs for these experiments were synthesized using the same RNA samples as in the microarray. The data were normalized relative to the gene expression values of the planktonic samples, which were arbitrarily assigned a value of 1 and are not shown in the graph. White bars are results from the "+/− saliva" experiments, and the bars shaded in gray are the results from the "saliva +/− lysine" experiments. Shown here is one representative data set. This experiment was performed three times with similar results each time.

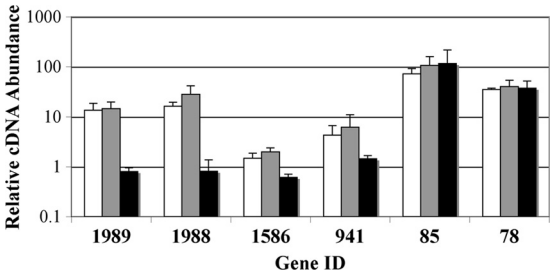

FIG. 7. Real-time RT-PCR of genes induced by centrifugation. Of the 12 genes tested from the autoaggregation microarray, 6 were found to be inducible by centrifugation. Cell pellets were incubated for 1 h after centrifugation before the RNA was extracted to be used for the RT-PCR analysis. The data are normalized relative to the gene expression values of the dispersed samples, which were arbitrarily assigned a value of 1 and are not shown in the graph. White bars are representative of gene expression in cell pellets incubated in SDM, gray bars represent data from cell pellets incubated in SDM plus 25% saliva, and black bars represent data from cell pellets incubated in SDM plus 25% saliva plus 50 mM lysine. Shown here are the averages of three independent experiments.

be an accurate representation of the overall trends in gene expression and in many cases are also likely to be a close reflection of the gene expression changes as measured by real-time RT-PCR.

Centrifugation can induce a similar response as autoaggregation. Given the large change in cell density that occurs as a result of autoaggregation, we were curious as to whether centrifuged cells would exhibit a similar gene response as aggregated cells. As described in Materials and Methods, we centrifuged 25586 using a low-speed spin and resuspended 1 set of samples in SDM. The cell pellets were incubated in either fresh SDM, SDM plus 25% saliva, or SDM plus 25% saliva plus 50 mM L-lysine. Since we previously assayed the response to ~1 h of autoaggregation, we incubated the dispersed and pelleted samples for 1 h before extracting RNA. As shown in Fig. 7, half of the genes we previously tested by RT-PCR were also responsive to 1 h of incubation as a cell pellet. As expected, we saw no evidence that saliva could specifically alter the expression pattern of any of the 12 genes we tested. Interestingly, L-lysine was still able to inhibit the induction of FN1989, FN1988, FN1586, and FN0941 despite the extremely high cell density of the cell pellets. However, this was not the case for both FN0085 and FN0078, which were similarly responsive to high cell density regardless of medium composition. FN0085 and FN0078 also exhibited a greater induction in cell pellets compared to precipitated autoaggregates (Fig. 6 and 7), which suggests that both genes probably exhibit greater expression with increasing cell density.

DISCUSSION

Autoaggregation seems to be a common phenomenon among oral bacterial isolates, although it appears to be largely strain specific. The species *F. nucleatum* is perhaps best known for its prodigious repertoire of coaggregations; however, little has been reported regarding the autoaggregation of this species. In *F. nucleatum* ATCC strain 25586, we found a rapid autoaggregation response in the presence of saliva but found no evidence for this ability in the closely related strain ATCC 23726. Recently, two separate reports examining multiple fresh clinical isolates of *F. nucleatum* both reported autoaggregation in their strains (20, 35), and a similar finding was recently reported with canine fusobacterial isolates as well (10). Thus, it may be that some laboratory strains of *F. nucleatum* have lost this ability upon subculturing, similar to what is routinely seen with *A. actinomycetemcomitans* (11, 18). Certainly, strain-specific differences in genetic content may also account for differences in autoaggregation. Surprisingly, we found autoaggregation in 25586 to be inhibited by lysine, similar to its coaggregation with streptococci (38). In addition, our microarray data suggest that autoaggregation impacts the expression of a large number of genes in a relatively short time frame. Since we assayed the effect of autoaggregation using two different conditions and found strikingly similar transcriptional responses, there is reason to suspect that these genes are indeed regulated by some signal(s) related to aggregation. Whether this signal occurs as a result of changes in nutrient availability, short-range gradients of diffusible molecules, or direct cell-cell contact remains to be determined. In addition, many of these same transcriptional responses could be recreated simply by centrifuging the cells and incubating them as a cell pellet. Interestingly, under these conditions, lysine was still able to function as an inhibitor. It should also be noted that the microarray data exhibited no changes in any typical stress response genes, suggesting the transcriptional responses were unlikely to be attributable to either starvation or toxicity derived from saliva or lysine. To the best of our knowledge, these are also the first microarray data for *F. nucleatum*.

Autoaggregation stimulates metabolic diversification. The data from the microarray experiments suggest that aggregation induces a rapid transcriptional response from numerous metabolic pathways in the cell. Based upon the large number of genes associated with either carbon source acquisition or catabolism, it appears that autoaggregation stimulates the cell to quickly diversify its repertoire of energy sources. For instance, the strongest induction occurred in two adjacent genes encoding a putative tyrosine phenol-lyase (FN1988) and a putative sodium-dependent tyrosine transporter (FN1989) (Fig. 6). Tyrosine phenol-lyases convert tyrosine into phenol, pyruvate, and ammonia. Thus, tyrosine is likely being utilized as a source of pyruvate in aggregated cells. Curiously, previous studies of amino acid preferences in *F. nucleatum* have suggested that tyrosine catabolism is strain specific (3, 13). Based upon our results, it may be that certain strains require aggregation in order to stimulate this ability. It would be interesting to determine whether aggregation-inducible tyrosine catabolism has any relationship with the statherin binding ability of *F. nucleatum* (40). Statherin is a 43-amino-acid tyrosine-rich pellicle protein that has been shown to promote *F. nucleatum* adherence to hydroxyapatite beads (19, 40).

In addition to tyrosine, serine metabolism appeared to be induced by autoaggregation. This ability is encoded in a three-gene operon comprised of a putative serine racemace (FN0552), D-serine dehydratase (FN0553), and an ABC transporter (FN0554), presumably for serine. Similar to tyrosine phenol-lyases, D-serine dehydratases generate pyruvate and ammonia. Glutamate is a preferred carbon source for *F. nucleatum* (8, 13), and the microarray data suggested that aggre-

gation induces a large number of genes that could provide alternative sources of glutamate. For example, FN0590 and FN0591 form a two-gene operon encoding a putative aminobenzoyl-glutamate transporter (FN0591) and an aminobenzoyl glutamate hydrolase (FN0590). We also observed increased transcription of FN1186, another potential aminobenzoyl glutamate hydrolase. A variety of peptidases predicted to cleave glutamate from peptides were induced, including FN0999 and FN1280 as well as a γ-glutamyltranspeptidase (FN0941), which is a characteristic *F. nucleatum* peptidase that generates glutamate from glutathione (6). A putative sodium glutamate symporter (FN1801) was induced, as were several key genes of the butyrate fermentation pathway (FN0271 to FN0273), which is the main endpoint for glutamate metabolism. The butyrate fermentation pathway also likely functions as the endpoint for the ethanolamine degradation pathway (FN0078 to FN0090), due to its generation of acetyl coenzyme A. It is still unclear what might be the main source of ethanolamine in the diet of *F. nucleatum* or why its utilization is stimulated by high cell density but, given the high concentration of ethanolamine in cellular membranes, it would be interesting to determine whether neighboring dead/lysed cells could serve as an energy source.

Sodium homeostasis in aggregated cells. Fusobacteria maintain a transmembrane electrochemical gradient of sodium to energize the reactions used in amino acid transport and glutamate catabolism, one of several features these species share with the clostridia (5, 17). As previously described, we observed induction in numerous genes related to both amino acid uptake and glutamate catabolism. Therefore, it is not surprising that we also observed a corresponding increase in genes related to sodium homeostasis. For example, FN1801 and FN1989 are both predicted sodium-dependent amino acid symporters, which increase the cytosolic concentration of sodium as a consequence of amino acid import. In order to maintain the required electrochemical gradient that facilitates these functions, *F. nucleatum* encodes a V-type ATPase that can hydrolyze ATP to extrude Na^+ ions from the cell. These genes are clustered into two adjacent operons (FN1733 to FN1742), both of which were induced. We also observed induction in three separate Na^+/H^+ antiporters (FN0650, FN1422, and FN2077). These proteins can utilize a proton gradient to extrude Na^+ in an ATP-independent fashion, but as the pH increases, the proton gradient dissipates and the antiporters lose functionality (16). Since amino acid fermentation yields ammonia and increases the local pH, particularly under high cell density conditions, it may explain why *F. nucleatum* requires a V-type ATPase system, despite having multiple Na^+/H^+ antiporters.

Antimicrobial resistance in aggregated cells. β-Lactamase production appears to be a common feature of clinical isolates of *F. nucleatum* (7, 14, 25, 27, 30, 31). Interestingly, we found that autoaggregation induced an operon containing one or two hypothetical open reading frames (FN1582 and FN1583) and a putative β-lactamase (FN1584). In vivo and in vitro data have suggested that β-lactamases are of particular concern in polymicrobial infections, since they can complement the susceptibility of nearby species (37). Given the ability of *F. nucleatum* to associate with a large number of other organisms, it is conceivable that *F. nucleatum* could afford protection to numerous species, such as the streptococci, which are both highly susceptible to β-lactams and typical coaggregation partners of *F. nucleatum*. We also observed increased transcription of FN0515 and FN0516, which are predicted efflux pumps used to confer resistance to a broad spectrum of antimicrobials. Thus, it appeared that autoaggregation stimulated the cellular defenses to a variety of xenobiotic substances.

Summary. From the results presented here, several inferences can be made regarding *F. nucleatum*. Strains such as 25586 are likely to become highly adherent in the presence of saliva, which seems logical, since free-floating cells in the saliva are doomed to clearance from the oral cavity. *F. nucleatum* has several options to precipitate out of solution and become immobilized onto an oral surface. These could include autoaggregation, coaggregation, adherence to the salivary pellicle, or perhaps combinations of each. Once the cells have precipitated and/or become immobilized, a tremendous change in cell density occurs due to the physical interactions between cells. Our data suggest that intraspecies interactions will stimulate the cells to increase their ability to transport and metabolize various carbon sources and increase their resistance to antimicrobial agents. We speculate that these could be some of the earliest transcriptional events that occur as *F. nucleatum* transitions itself to a biofilm-dwelling organism. Studies are currently under way to determine whether coaggregation elicits a similar transcriptional response from *F. nucleatum*.

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